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Reflectivity from floating bilayers: can we keep the structural asymmetry?

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Abstract. To assess the structure of complex biomembranes, the use of asymmetric model systems is rare, due to the difficulty of realizing artificial membranes with desired heterogeneous composition and applicable for single membrane structural investigation. We developed an experimental model with a single macroscopic bilayer floating on top of another adhering to a silicon flat surface, prepared by Langmuir-Blodgett Langmuir-Schaefer technique, then investigated by neutron reflectivity. On the way to more complex systems, containing lipids of different nature, we tested whether a simple imposed asymmetry is kept in time and whether it can stand some standard experimental protocols commonly employed in treating model membranes. We focused on cholesterol, a basic component with a transverse distribution that is not symmetric in biomembranes, and may assume specific location in functional domains. So we forced different asymmetries in the "adhering + floating" bilayers system composed of phospholipids and cholesterol in bio-similar mole ratios. The neutron reflection accessible length-scale and its sensitivity, enhanced by the possibility to play with deuteration, allowed assessing the cross profile of the membrane and revealing that lipid redistribution can occur.

1. Introduction

The structural complexity of biomembranes, based on their heterogeneity in composition, dramatically involves the asymmetric disposition of different components, from lipids to proteins, in the transverse or the longitudinal directions. Furthermore, inhomogeneities in the two leaflets of a membrane can couple, constituting the basis for the structural stabilization and modulation of functional domains involved in transmembrane signaling. In order to build suitable mimics of complex biomembranes, it is then necessary to realize asymmetric model systems applicable for structural investigation. The experimental study of asymmetric model membranes is rare, due to the difficulty of realizing artificial membranes with wanted and defined heterogeneous composition. However some attempts have been made, for example by preparing phospholipid unilamellar vesicles (LUVs) containing small amounts of glycosphingolipids only in their outer layer. The mechanical properties of the membrane were found to be strongly affected by the doping glycolipids, producing a softening that turns to hardening in the case of symmetric redistribution of molecules [1]. Fine assessment of the inner structure and

inhomogeneities of the membrane requires overall aggregate monodispersity. In this framework we developed an experimental model with a single macroscopic bilayer floating at 1.5-2 nm on top of another adhering to a silicon flat surface, prepared, layer-by-layer, by combination of Langmuir-Blodgett and Langmuir-Schaefer techniques. The system so built has led in the past to stable and reproducible floating bilayers [2]. With this technique the composition of each layer can be chosen suitably. On the way to more complex systems, containing lipids of different nature, we tested whether a simple imposed asymmetry was kept in time and whether it could stand some standard experimental protocols commonly employed when dealing with model membranes (i.e. annealing and solvent exchange). We focused on cholesterol, a basic component with a transverse distribution that is not symmetric in biomembranes, and may assume specific location in functional asymmetric domains. It has been shown that floating bilayer systems containing cholesterol can be prepared, obtaining stable membranes for low cholesterol:phospholipid ratios, in the range 1-10 mol % [3-9].

In the present work, we forced asymmetry in an "adhering + floating" bilayers system composed of phospholipids and cholesterol in much higher amounts, in bio-similar mole ratios (11:2.5 mol:mol). We investigated the structure of the bilayers by neutron reflectivity, which couples the Angstrom scale of neutron accessibility to the possibility of changing the contrast profile of the system. In fact, selective deuteration of lipids or different H_2O/D_2O solvent composition can be exploited to allow or enhance the visibility of different components.

We present results on the feasibility of the desired asymmetric free floating membranes, and on the effects of common experimental protocols on the overall stability of the bilayer and on the lipid redistribution between different leaflets.

2. Materials and methods

Both hydrogenated and fully deuterated lipids were used. Cholesterol was purchased from Sigma-Aldrich Co, d_{85}-DSPC, d_{75}-DPPC were from Avanti Polar Lipids Co.. According to a well assessed standard protocol [10-11], lipids were dissolved in chloroform (99%) to a final concentration of 1mg/ml. Mixed lipid systems were obtained by mixing appropriate amounts of various chloroform lipid solutions. Lipids were then deposited on the surface of a Langmuir trough filled with pure water kept at T = 18°C. The various monolayers were compressed un to a surface pressure of 40 mN/m, similar to the one in real systems [2], while recording the corresponding (π-A) isotherms. All of the used monolayers are in the gel phase in these conditions. They were then layer-by-layer deposited on a silicon substrate. Asymmetric bilayers were realized by completely changing the monolayer in between different steps.

Pressure-area isotherms

Pressure-area (π-A) isotherms were recorded on a Nima Langmuir-Blodgett trough, using a Wilhelmy plate for pressure sensing. All (π-A) experiments were carried out at 18°C (±0.5), below the melting temperature of the lipids used. Water for the subphase was processed in a Milli-Q system (Millipore, Bedford, MA), to a resistivity of 18 MΩ.cm. Each lipid solution was spread over the water subphase and chloroform was let to evaporate completely, over 15min. All isotherms were recorded using a barrier speed of 25 cm^2/min.

Stability and reproducibility of Langmuir films were verified by performing various compression-expansion cycles on two different Nima Langmuir troughs.

Floating bilayer buildup

Substrates were single crystals of silicon (5 x 5 x 1.5 cm^3) polished on one large face (111) . The silicon blocks were cleaned before use in subsequent baths of chloroform, acetone, ethanol, water and treated with UV-Ozone for 30min [12]. The contrast solutions were H_2O (Milli-Q system), D_2O (99% pure, provided by ILL) and Silicon Matched Water, that is, a mixture of H_2O and D_2O with the same scattering length density of Silicon (SMW i.e. 0.62 H_2O and 0.38 D_2O volume fractions).

Double bilayer depositions were done in water coupling the Langmuir-Blodgett [13] and Langmuir-Schaefer Techniques [14], as follows. At the initial stage, the silicon block was immersed in water at 18°C in the Langmuir trough. A suitable lipid solution, the one chosen for the adhering bilayer was spread on the water surface and progressively compressed to 40 mN/m, the optimal for phosphocolines [2]. The silicon block was then almost completely risen and subsequently dipped again into water (speed 6 mm/min) across the monolayer, keeping the pressure constant. Two facing monolayers were adsorbed onto the block. Then, the water surface was completely cleaned and a different solution was spread, with the composition desired for the inner side of the floating membrane. The third layer was deposited by rising the block again, according to the same Langmuir technique. To prepare asymmetric floating bilayers, the surface of the trough was cleaned again and a solution with the composition desired for the outer side of the floating membrane was spread and compressed to 40 mN/m. The fourth closing monolayer was deposited by rotating the block by 90°, in the Langmuir Schaefer configuration, and lowering it carefully onto the surface.

The block was then closed in a teflon cell and fixed by an aluminum thermostated cage.

Neutron Reflectivity

Reflectivity measurements were performed on the D17 [15] reflectometer at ILL, Grenoble, France (TOF mode, resolution = 1-5%, λ range between 2 and 20 Å, with two incoming angles of 0.7 and 4°).The cell was oriented vertically and kept in position while changing solvents and temperature. Measurements were performed at the silicon-water interface, the beam coming from the side of the silicon block.

In a neutron reflectivity measurement R, the ratio between the intensities of the reflected and incoming beams, is collected, as a function of q, the momentum transfer perpendicular to the interface [16].

Reflectivity is related to the scattering length density across the interface by the approximate relation:

$$R(q_z) \approx \frac{(16\pi^2)}{q_z^2}\left|\rho(q_z)\right|^2$$

which is the reflectivity in the Born approximation [17]. $\rho(q_z)$ is the Fourier transform of the scattering length profile $\rho(z)$ along the normal to the interface, giving information about the composition of each layer and about its local structure. The scattering length density is given by:

$$\rho(z) = \sum_j b_j n_j = \sum_j b_j n_j$$

where n_j is the number of nuclei per unit volume and b_j is the scattering length of nucleus j.

The method of analysis often used for specular reflection data involves the construction of a model of the interface that may be represented by a series of parallel layers of homogeneous material. Each layer is characterised by a scattering length density (SLD) and a thickness, which are used to calculate a model reflectivity profile by means of the optical matrix method (Born and Wolfe, 1989). The interfacial roughness between any two consecutive layers may also be included in the model by the Abeles method. The calculated profile is compared to the measured profile and the quality of the fit is assessed by using χ^2 in the least-squares method.

Data were analyzed using the software Motofit [18], allowing simultaneous fit of data sets referred to the same sample in different contrast conditions, using the SLDs reported in table 1.

Table 1. Properties of materials used

Material	SLD (10^{-6}Å^{-2})[a]
Si	2.07
SiO_2	3.41
H_2O	-0.56
D_2O	6.36
cholesterol $C_{27}H_{46}O$	0.22
deuterated lipid heads $C_{10}D_{13}H_5O_8PN$	5.70
deuterated lipid chains $C_{30}D_{62}$ gel phase	7.66
deuterated lipid chains $C_{30}D_{62}$ fluid phase	6.13

[a] SLD values are from ref [19] and [20]

3. Results and discussion

3.1 Langmuir monolayers

Since the final goal of our study is to assess the structure of complex biomembranes, with this work we want to build up a basic model membrane with defined heterogeneous and asymmetric composition.

The effects generated by cholesterol addition to floating membranes have been studied [3-4] by means of neutron reflectivity in the case of low total amount of cholesterol, with particular attention to the phase behavior.

We now want to build up stable asymmetric floating cholesterol-containing bilayers with bio-similar composition. The aim of this work was to study the cholesterol transverse distribution inside the membrane and to test whether the forced asymmetry is kept.

First, we verified the effect brought about by addition of cholesterol to DPPC, by recording the (π-A) curves of the mixed Langmuir monolayers at the air-water interface as a function of different molar ratios between d_{75}-DPPC and cholesterol, from the gas to gel phase and, particularly, to the 40 mN/m pressure required for deposition. The corresponding curves are reported in fig 2 and display the expected features, following the existing literature. This guarantees the original integrity and compactness of the bilayer-forming monolayers. Moreover, the monolayers are stable in time and the pressure-area results are repeatable both after subsequent compression-expansion cycles and after monolayer re-spreading.

Progressive addition of cholesterol to d_{75}-DPPC results in progressively decreasing the occupied area per average molecule. For all the systems, at a given pressure, the measured area per molecule is lower than expected for ideal mixing. In fact, the effect of cholesterol is to rigidify and order the lipid chains, with a consequent reduction of the occupied area per average molecule at the air-water interface.

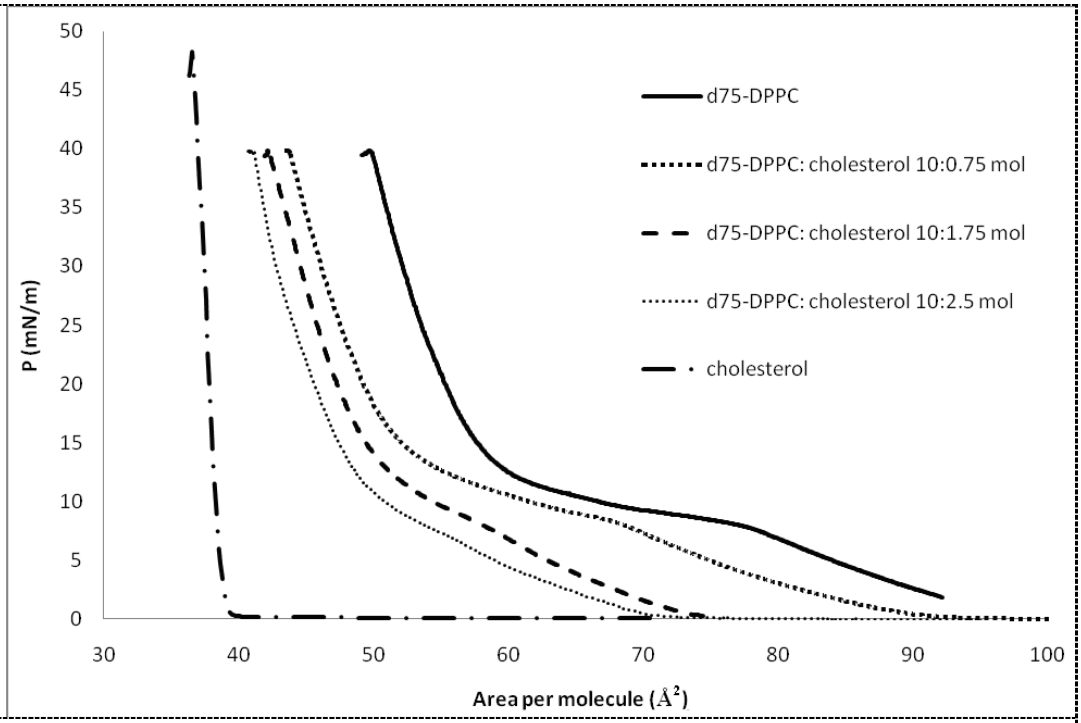
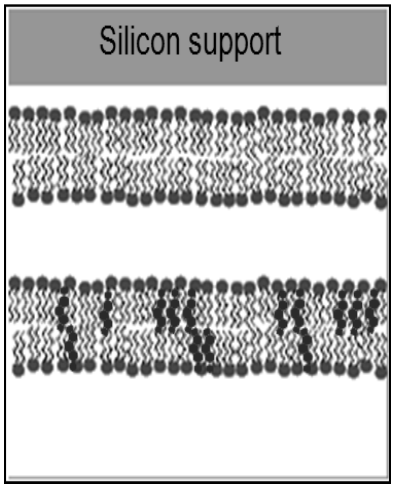


Figure 1. Pressure-area isotherms of various mixtures of d_{75}-DPPC and cholesterol at 18°C. The subphase is water.

To study the cholesterol transverse distribution inside the membrane we compared two floating model membranes: one was a 'blank', with no cholesterol, the other was a cholesterol-containing bilayer (see fig 2). For the bilayer adhering to the silicon support, we selected the long chain phospholipid DSPC, being in gel phase all over the investigated temperature range, from 22°C to 51°C. This guarantees the compactness and stability of the supporting bilayer. For the floating bilayer, DPPC was used as lipid matrix, being the lipid most likely to be found in membrane domains enriched in cholesterol and sphingolipids.

In biological membrane domains, the three components phospholipid : sphingolipid : cholesterol are present in a 10 : 1 : 2.5 molar ratio. A good approximation to simulate the composition of a biomembrane microdomain is obtained by inserting the 70% of the total amount of cholesterol in the inner layer of the membrane, and 30% in the outer. Accordingly, as reported in fig 1, for the cholesterol-containing system, the third layer was made of DPPC : cholesterol in molar ratio 11 : 1.75 and the fourth layer was made of DPPC : cholesterol in molar ratio 11 : 0.75.

We also applied selective deuteration to enhance or minimize the visibility of different components of the membranes under study. In particular, deuterated d_{75}-DPPC was used in order to highlight cholesterol with respect to the lipid matrix.



		Sample 1	Sample 2
Layer 1	ADHERENT BILAYER	DSPC	d_{85}-DSPC
Layer 2		DSPC	d_{85}-DSPC
Layer 3	FLOATING BILAYER	d_{75}-DPPC	d_{75}-DPPC:CHOL 11:1.75
Layer 4		d_{75}-DPPC	d_{75}-DPPC:CHOL 11:0.75

Figure 2. Scheme of the samples construction

3.2 Reflectivity measurements

Measurements on a bare silicon block were performed, in different solvents (H_2O, D_2O, SMW), to measure the characteristics of the silicon oxide layer formed at the silicon-air interface.

Then, both the bare matrix and the cholesterol containing samples were placed under the neutron beam as they came from the deposition procedure, without any annealing. They were thermostated at 22°C and four subsequent reflectivity measurements were taken, of one hour each, to evaluate any molecular exchange eventually occurring among the four lipid layers. The four spectra overlap, showing that the system is stable in these conditions over these times.

As a further step, the cholesterol containing system was submitted to a temperature increase to 51°C (the procedure currently meant by annealing) and, after a reflectivity measurement, it was lowered back to 22°C. Reflectivity was measured again at this point. Results are reported in fig 3.

Experiments at 22°C, after the annealing, have been performed in three contrast solutions: water, heavy water and silicon matched water. The adhering + floating system has shown to be stable against solvent exchange, consisting of progressive substitution of the aqueous solvent under continuous flow. This opens the possibility to use a biological relevant solvent in a true biomimetic perspective.

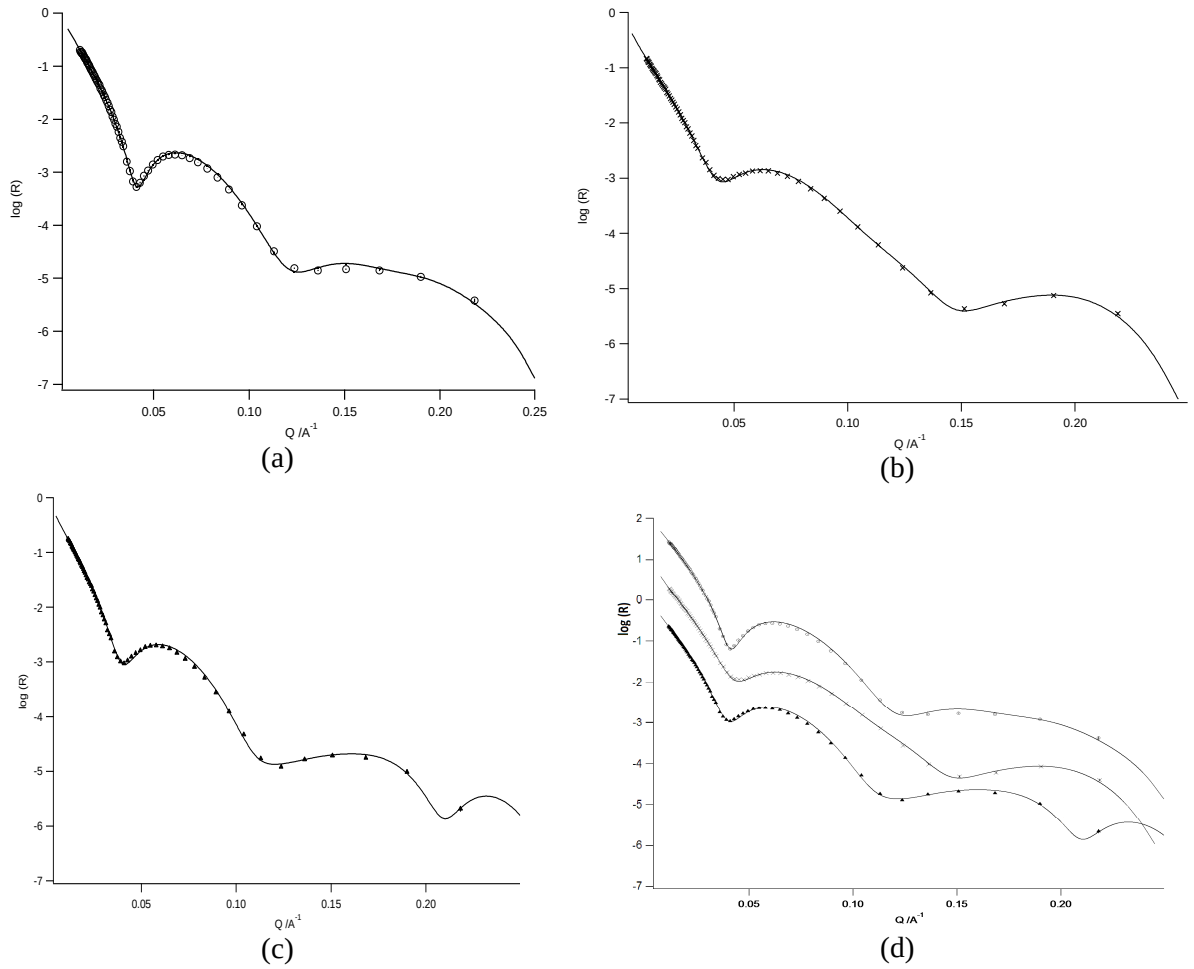


Figure 3. Reflectivity spectra of the cholesterol-containing sample in H_2O (a) at 22°C before annealing, (b) at 51°C, (c) at 22°C after annealing.

In insert (d) the curves have been arbitrarily shifted by 5 (51°C) and 10 (22 °C) for better clarity in comparing them: upper curve: 22°C before annealing. Middle curve: 51°C. Lower curve: 22°C after annealing.

The fit of the experimental data was performed with Motofit using a 11-layers model. This was composed by the silicon oxide, a water layer, the first bilayer (composed of 4 layers: heads, chains, chains, heads), a water layer and the floating bilayer (composed of 4 layers: heads, chains, chains, heads). We performed a simultaneous fit of the three solvents, gaining a better resolution for the complex structure. The obtained parameters are reported in tables 2 and 3. The errors are estimated from the maximum variation in the acceptable fit subject to the constraints of space filling and stoichiometry.

First, from table 2, we notice that the addition of cholesterol leads to a thickening of the floating membrane, despite a reduction of water content. This indicates that the bilayer is more compact, in agreement with the (π-A) curves. Also, it increases the roughness among the layers, suggesting that some cholesterol molecules can sit across the layers, without participating exclusively to either of

them. This effect has been observed also in DPPC- cholesterol supported bilayers [21]. The cholesterol presence in the two layers seems not uniform, although not respecting the forced distribution. No significant differences between the two adherent DSPC bilayers are detected. No water was detected between the silicon oxide and the first bilayer headgroup, whereas between the two bilayers a 20±1 Å thick water layer was found.

Table 2. Parameters obtained fitting the reflectivity profiles of two samples studied. Heads and chains 3 refer to the inner layer of the floating bilayer, 4 refer to the outer layer.

	d_{75}-DPPC			**d_{75}-DPPC + chol**		
	t; r [a]	w [b]	chol [c]	t; r [a]	w [b]	chol [c]
Heads 3	8; 4	20	0	7; 1	15	0
Chains 3	16; 5	12	0	22; 8	6	43
Chains 4	18; 1	19	0	18; 8	11	57
Heads 4	9; 5	25	0	9; 4	16	0

[a] t=layer thickness (±1, Å); r= layer roughness (±2 Å).

[b] w= layer water content (±5 % volume).

[c] chol= layer cholesterol relative content (±2% over the total amount of cholesterol i.e. 11:2.5 molar ratio lipid:cholesterol).

Table 3. Parameters obtained fitting the reflectivity profiles at different temperatures of the asymmetric cholesterol containing sample. The numeration of layers goes from 1 to 4, starting from the layer towards the Silicon to the outer layer of the floating membrane.

	22°C			**51°C**			**22°C back**		
	t; r [a]	w [b]	chol [c]	t; r [a]	w [b]	chol [c]	t; r [a]	w [b]	chol [c]
Heads 1	10; 8	24	0	9; 8	22	**0**	6; 8	15	0
Chains 1	19; 1	17	0	19; 1	15	0	22; 6	7	0
Chains 2	19; 1	19	0	23; 8	10	0	23; 5	21	0
Heads 2	8; 8	25	0	7; 8	19	0	8; 5	25	0
water	19; 8		0	22; 3		0	20; 1		0
Heads 3	7; 1	15	0	6; 8	45	0	10; 8	45	0
Chains 3	22; 8	6	43	16; 8	38	58	16; 9	40	50
Chains 4	18; 8	11	57	16; 1	28	42	16; 7	40	50
Heads 4	9; 4	16	0	6; 1	45	0	10; 9	45	0

[a] t=layer thickness (±1, Å); r= layer roughness (±2 Å).

[b] w= layer water content (±5 % volume).

[c] chol= layer cholesterol relative content (±2% over the total amount of cholesterol i.e. 11:2.5 molar ratio lipid:cholesterol).

A different situation is found upon annealing, as shown in table 3.

No cholesterol was found in the d_{85}-DSPC adhering bilayer before, during or after the annealing. However the cholesterol was found to assume a symmetric distribution in the hydrophobic region of the floating d_{75}-DPPC bilayer. This indicates that there is no structural stringent constraint for cholesterol as such to stay in an asymmetric distribution. Kinetic or trapping effects could be involved in the initial asymmetry. Also, at 51°C, over the floating bilayer melting temperature, the water layer between the two bilayers thickens from 20 to 22 Å.

Annealing the sample is commonly used to reduce local packing defects originated by the preparation procedures of lipid systems, and then it is applied also to deposited bilayers. Data of Table

3 show that the first bilayer is presumably extensively affected by single-lipid protrusion. In fact, the strictly-hydrophobic region is shallower than expected for a DSPC bilayer, while the shells are thicker, rougher and more hydrated. After annealing, the structural parameters of the first bilayer become closer to expected, and it becomes more compact. On the other hand, after annealing, the boundaries of the different regions of the floating bilayer become more uniform and defined. But we show that annealing is likely to induce also a more extensive redistribution of lipids, impeding the realization of biomimetic systems of desired asymmetry. It can be seen that after annealing the distribution of cholesterol has been flattened, within the floating bilayer. In these conditions as reasonable no cholesterol migration between bilayers has occurred. We also observe that after annealing the hydration of the outer three layers increases. The floating bilayer chains contain 40% in volume of water and its thickness decreases.

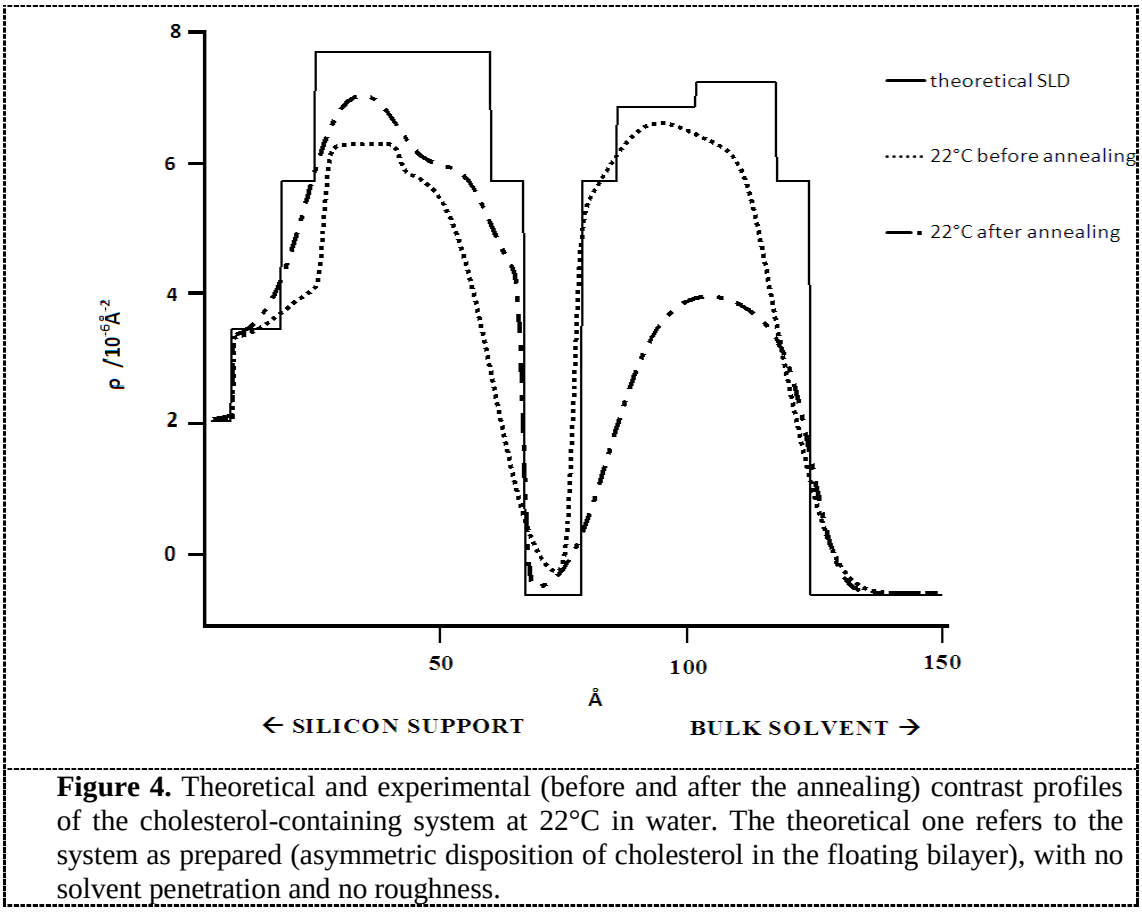


Figure 4. Theoretical and experimental (before and after the annealing) contrast profiles of the cholesterol-containing system at 22°C in water. The theoretical one refers to the system as prepared (asymmetric disposition of cholesterol in the floating bilayer), with no solvent penetration and no roughness.

The present experiment suggests that annealing should eventually be excluded from the protocol to be used with asymmetric samples, both for lipid redistribution and for macroscopic bilayer integrity. In fact, the estimated water penetration after annealing could correspond to bilayer punctuation or fragmentation occurring at high temperature.

On the other hand, some positive indication can be drawn. The role of coupling of molecules, shapes and morphology, in the build-up and in the non-covalent stabilization of uneven distribution of components is a debated topic. This is likely to show up even in the simplest cases, as the one presented here. In real membranes segregation, preferential distribution and domain formation are existing phenomena, also strongly involving cholesterol. Alternatively, this experiment shows that cholesterol is easily evenly redistributed in the forcedly flat, bicomponent membrane considered, despite the initial asymmetry. This seems to redirect to the concept of coupling as determinant for

membrane structure: coupling between membrane curvature and molecular sorting, as well as coupling of pair molecules matched in the packing shape. Cholesterol uneven distribution could be preserved in a non-flat geometry or by a suitable shape-coupling with other lipids within a complex membrane (like glycosphingolipids) also without invoking a prominent role of proteins. In addition, the characteristic umbrella effect, typical of cholesterol and evident in the π-A behaviour, could be modulated by 3D morphological constraints.

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